PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/537, 33/543, 33/58

A1

(11) International Publication Number: WO 94/12878

(43) International Publication Date: 9 June 1994 (09.06.94)

(21) International Application Number: PCT/GB93/02454

(22) International Filing Date: 29 November 1993 (29.11.93)

(30) Priority Data:

9224897.0	27 November 1992 (27.11.92)	GB
9224898.8	27 November 1992 (27.11.92)	GB
9315991.1	2 August 1993 (02.08.93)	GB
9315995.2	2 August 1993 (02.08.93)	GB
9319298.7	17 September 1993 (17.09.93)	GB

(71) Applicant *(for all designated States except US)*: GEC-MARCONI LIMITED [GB/GB]; The Grove, Warren Lane, Stanmore, Middlesex HA7 4LY (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: ABUKNESHA, Ramadan, Arbi [LY/GB]; 16 Burgess Park Mansions, Fortune Green Road, London NW6 1DP (GB).

(74) Agent: COCKAYNE, Gillian; GEC Patent Dept., Waterhouse Lane, Chelmsford, Essex CM1 2QX (GB).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: SEPARATION METHOD

(57) Abstract

The present invention relates to a separation method which finds application in immunological detection (e.g. in immunoassay and immunosensors), to a method suitable for use in detection, to a sensor, to apparatus for use in detection, and to a test-kit. The invention provides, *inter alia*, a separation method, suitable for use in an immunological method for the detection of a plurality of entities, which separation method includes the use of an auxiliary species provided on a support material and the use of an additional immunological species (as defined in the Specification) provided on a support material. The invention finds applicationin "multi-entity detection" (e.g. "multi-analyte species detection").

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Separation Method

The present invention relates to a separation method which finds application in immunological detection (e.g. in immunoassay and immunosensors), to a method suitable for use in detection, to a sensor, to apparatus for use in detection, and to a test-kit.

According to one aspect of the present invention there is provided a separation method, suitable for use in an immunological method for the detection of a plurality of entities, which separation method includes the use of an auxiliary species provided on a support material and the use of an additional immunological species (as hereinafter defined) provided on a support material.

According to another aspect of the present invention there is provided a method, suitable for use in immunological detection of a plurality of entities, which method includes the use of an auxiliary species provided on a support material and the use of an additional immunological species (as hereinafter defined) provided on a support material.

According to a further aspect of the present invention there is provided a sensor, suitable for use in immunological detection of a plurality of entities, which sensor includes an auxiliary species and an additional immunological species (as hereinafter defined), said auxiliary species and said additional immunological species being provided on a support material.

According to a further aspect of the present invention there is provided apparatus, suitable for use in immunological detection of a plurality of entities, which apparatus includes an auxiliary species and an additional immunological species (as hereinafter defined), said auxiliary species and said additional immunological species being provided on a support material or support materials.

According to yet a further aspect of the present invention there is provided a test-kit, suitable for use in immunological detection of a plurality of entities, which test-kit includes an auxiliary species and an additional immunological species (as hereinafter defined), said auxiliary species and said additional immunological species being provided on a support material, or support materials.

The auxiliary species may be, for example, an auxiliary ligand or an auxiliary binder.

The additional immunological species is a species capable of undergoing specific binding with a reaction partner therefor (which partner will be hereinafter be referred to as an additional immunological species reaction partner). In accordance with the present invention the additional immunological species either is a second antibody (e.g. an antibody which is capable of binding with a primary antibody), or is a binder (e.g. an antibody) capable of binding with a binder of a selected auxiliary ligand-binder system (e.g. an auxiliary ligand-auxiliary binder system), or is a primary species (e.g. a ligand (such as a hapten (e.g. a hapten derivative) or an antigen or an antibody), or is a binder (e.g. an antibody) capable of binding with a complex which complex is a complex of an auxiliary species and an auxiliary species reaction partner (e.g. an auxiliary ligand and a binder (e.g. an auxiliary binder)) (which complex may be, for example, an immunological complex or a non-immunological complex), or is a binder capable of binding

part of a bifunctional antibody or capable of binding a complex which includes a bifunctional antibody, or is a species of like nature.

An auxiliary species may be such that it is capable of undergoing a specific binding reaction with a reaction partner therefor (which partner will be hereinafter referred to as an auxiliary species reaction partner).

Where an auxiliary species is an auxiliary ligand, an auxiliary species reaction partner may be a binder for the auxiliary ligand.

Conversely, where an auxiliary species is an auxiliary binder, an auxiliary species reaction partner may be an auxiliary ligand.

A binder for a given auxiliary ligand may be a species capable of undergoing a specific binding reaction with the auxiliary ligand. Where, for example, the binder is an auxiliary species, as is further disclosed hereinafter, the binder may be an auxiliary binder as is also further disclosed hereinafter. Alternatively, for example, the binder may be a species which has a part which is capable of undergoing a specific binding reaction with the auxiliary ligand and a part which is capable of undergoing a specific binding reaction with a primary species, as is further disclosed hereinafter.

It is to be understood that a separation method in accordance with the present invention may find application in, for example, separation of fractions in an immunoassay method.

By way of example, by use of an auxiliary species and an additional immunological species, or by use of a plurality of different auxiliary species and an additional immunological species, or by use of an auxiliary species and a plurality of different additional immunological species, or by use of a plurality of different auxiliary species and a plurality of different additional immunological species, a plurality of different specific separations may be effected.

Accordingly, for example, a separation method in accordance with the present invention may involve a plurality of different specific separations. Thus, for example, by use of a plurality of separations in accordance with the present invention a plurality of different entities may be detected. Accordingly, the present invention may be utilised, for example, to effect "multi-entity" detection (e.g. "multi-analyte species" detection), as is further disclosed hereinafter.

An auxiliary species may be considered "auxiliary" if it does not take part in a primary immune binding reaction.

Thus, a ligand or a binder which does not take part in a primary immune binding reaction may be regarded, respectively, as an auxiliary ligand or an auxiliary binder.

By way of example, a primary immune binding reaction in accordance with the present invention is one in which an entity to be detected undergoes a specific binding reaction, or an authentic entity to be detected (as hereinafter defined) undergoes, a specific binding reaction, or an entity to be detected and an authentic entity to be detected undergo specific binding reactions. (It will be appreciated that the entity to be detected and the authentic entity to be detected undergo specific binding reactions with other species and not with each other.)

It is to be understood that an "authentic entity" is an entity which is capable of reacting in a substantially similar manner as an entity to be detected under substantially similar conditions. Further, it is to be understood that an authentic entity may, for example, have a chemically defined structure and/or a biologically defined activity.

It will be appreciated that an authentic entity to be detected may be used, for example, as a "standard" or a "calibrator". Where, for example, the entity to be

detected is itself an analyte species (as hereinafter disclosed) the authentic entity may be an authentic analyte species (or a suitable derivative thereof).

By way of example, in accordance with the present invention, specific binding between the auxiliary species and an auxiliary species reaction partner, and specific binding between additional immunological species and an additional immunological species reaction partner may be used to effect a separation in accordance with the present invention.

Thus, for example, a reaction partner for a given auxiliary species may be capable of being associated with a primary species such that upon binding between the auxiliary species and the reaction partner for the auxiliary species, primary species become associated (indirectly) with the support material.

Also, an additional immunological species may be used, in combination with an additional immunological species reaction partner to associate primary species with a support material.

For example, where the additional immunological species is a second antibody, a primary species may be associated with a support material by means of binding occurring between the second antibody and a primary antibody to which the second antibody is a binder; in these circumstances the primary antibody may be regarded as an additional immunochemical species reaction partner.

By way of further example, where the additional immunochemical species is a binder capable of binding a binder of a selected auxiliary ligand-binder system (e.g. an auxiliary ligand-auxiliary binder system) the binder of the selected auxiliary ligand-binder system may be regarded as an additional immunochemical species reaction partner and association of a primary species (which may be indirectly associated with the additional immunological species reaction partner) with the support material may be effected by binding between the

additional immunochemical species and the binder of the selected auxiliary ligand-binder system.

Further, by way of example, where the additional immunochemical species is a primary species an additional immunological species reaction partner may be another primary species; thus, for example, where the additional immunological species is a primary species comprising a ligand, the additional immunological species reaction partner may be a primary antibody, and conversely where the additional immunological species is a primary species comprising a primary antibody, the additional immunological species reaction partner may be a primary species comprising a ligand.

By way of further example, where the additional immunochemical species is a binder (e.g. an antibody) capable of binding a complex which complex is a complex of an auxiliary species and an auxiliary species reaction partner, the complex may be regarded as an additional immunological species reaction partner and association of a primary species (which may be associated with the complex in any suitable manner) with a support material may be effected by binding between the additional immunological species and the complex.

By way of further example, where the additional immunological species is a binder (e.g. an antibody) capable of binding a part of a bifunctional antibody or capable of binding a complex which includes a bifunctional antibody, a primary species (which may be associated in any suitable manner with the bifunctional antibody or with the complex which includes the bifunctional antibody) may become associated with a support material by binding between the additional immunological species and the part of the bifunctional antibody or binding between the additional immunological species and the complex.

By way of example, where the additional immunological species is a primary antibody, the antibody

may be such to permit a competitive immunoassay to be effected or such as to act as an "immobilising" primary antibody in, for example, a non-competitive "sandwich" immunoassay.

It will be appreciated that, by way of example, any suitable combination of auxiliary species and additional immunological species may be used in accordance with the present invention. For example, one type, or a plurality of types, of auxiliary ligands may be provided on a support material and one type, or a plurality of types, of additional immunological species may be provided on a support material.

Alternatively, by way of example, one type, or a plurality of types, of auxiliary binder may be provided on a support material and one type, or a plurality of types, of additional immunological species may be provided on a support material. By way of further example, one type, or a plurality of types, of auxiliary ligand and one type, or a plurality of types, of auxiliary binder and one type, or a plurality of types, of additional immunological species may be provided on a support material.

Thus, for example, it will be appreciated that although as few as one type of auxiliary ligand and/or one type of auxiliary binder may be provided on a support material and one type of additional immunological species may be provided on a support material, any suitable selected combination of types of auxiliary ligands and/or types of auxiliary binders and types of additional immunological species may be utilised.

It will be appreciated, for example, that if required, only one type of auxiliary ligand and one type of additional immunological species may be provided on a support material, or one type of auxiliary binder and one type of additional immunological species may be provided on a support material. However, in view of the foregoing it will be appreciated that, for example, any suitable

desired number of types of auxiliary ligands and/or auxiliary binders and additional immunological species may be provided on a support material as required.

Examples of auxiliary ligands and auxiliary binders (and binders having an auxiliary function) given hereinafter may find application as auxiliary species or auxiliary species reaction partners, or as part of a selected auxiliary ligand-binder system (e.g. auxiliary ligand-auxiliary binder system). Also, by way of example, a binder having one auxiliary function or a binder having two auxiliary functions may find application as an auxiliary species reaction partner. (An example of a binder having more than one function is a bifunctional antibody.) Where, for example, an additional immunological species is used which is capable of binding with a binder of a selected auxiliary ligand-binder system (e.g. the auxiliary binder of an auxiliary ligand-auxiliary binder system) it is to be understood that the auxiliary ligand and binder of the selected auxiliary ligand-binder system may be chosen such that the auxiliary ligand of such a system is different from any auxiliary ligand provided as an auxiliary species on a support material and different from any auxiliary ligand used as an auxiliary species reaction partner, and such that the binder of such a system is different from any binder provided as an auxiliary species on a support material and different from any binder used as an auxiliary species reaction partner.

An auxiliary ligand (e.g. for use as an auxiliary species or as an auxiliary species reaction partner or as an auxiliary ligand of a selected auxiliary ligand-auxiliary binder pair) may be any suitable ligand examples of which are antigenic ligands (such as haptens) and non-antigenic ligands.

Examples of antigenic ligands are 2,4 dinitrophenol, fluorescein, digitoxin, coumarin, cibacron blue, 2-(4-aminophenyl)-6-methyl benzothiazole-hemiglutarate,

camphorcarboxylic acid, 4-amino-benzo-15-crown-5, carboxyfluorescein, 3-methyl-1-adamantane acetic acid, 2-phenyl-4-quinoline carboxylic acid, xanthine-9-carboxyamide-glycine-glycine, 4-hydroxy-7-trifluoromethyl-3-quinaldine carboxylic acid, cis-bicyclo [3,3,0] octane-2-carboxylic acid, endo-bicyclo [2,2,2] oct-5-ene-2,3-dicarboxylic anhydride, N-[4-(4-aminobenzyl) phenyl]-5-norbornene-2,3-dicarboximide, and [IR-(2-endo, 3-exo)]-3-hydroxy-4,7,7-trimethyl bicyclo [2,2,1] heptane-2-acetic acid.

Examples of non-antigenic ligands are ligands of specific ligand-binder pairs (e.g. the ligand biotin in the case of the ligand-binder pair biotin-avidin).

Further examples of auxiliary ligands which may be attached to a support material (e.g. by covalent linkage or non-covalent attachment (adsorption)) are soluble materials for example soluble molecules (e.g. polymers) such as polypeptides, proteins, polysaccharides and conducting polymers; haptenic functions may be covalently coupled to such soluble materials (e.g. polymers). By way of example, an auxiliary ligand may be provided as a coating of a polymer on a support material.

A binder for an auxiliary ligand may be any suitable species capable of binding with an auxiliary ligand.

By way of example, the binder may be a binding protein (e.g. an antibody or a binding partner of a specific ligand-binder pair).

Thus, for example, where the auxiliary ligand is an antigenic ligand, the binder therefor may be an auxiliary binder which is an antibody to the ligand. An antibody to the auxiliary ligand may be considered to be an anti-auxiliary ligand antibody.

Examples of binders for antigenic ligands are anti-2,4 dinitrophenol antibody, anti-fluorescein antibody, anti-digitoxin antibody, anti-coumarin antibody, anti-cibacron blue antibody, anti-2-(4-aminophenyl)-6-methyl benzothiazole-hemiglutarate antibody, anti-

camphorcarboxylic acid antibody, anti-4-amino-benzo-15-crown-5 antibody, anti-carboxyfluorescein antibody, anti-3-methyl-1-adamantane acetic acid antibody, anti-2-phenyl-4-quinoline carboxylic acid antibody, anti-xanthine-9-carboxyamide-glycine-glycine antibody, anti-4-hydroxy-7-trifluoromethyl-3-quinaldine carboxylic acid antibody, anti-cis-bicyclo [3,3,0] octane-2-carboxylic acid antibody, anti-endo-bicyclo [2,2,2] oct-5-ene-2,3-dicarboxylic anhydride antibody, anti-N-[4-(4-aminobenzyl) phenyl]-5-norbornene-2,3-dicarboximide antibody, or anti-[IR-(2-endo, 3-exo)]-3-hydroxy-4,7,7-trimethyl bicyclo [2,2,1] heptane-2-acetic acid antibody.

Where, for example, an auxiliary ligand is a non-antigenic ligand, the ligand may be, for example, such that the binder is an auxiliary binder which is a binding partner that is a non-immunoglobulin (e.g. a naturally-occurring protein); the binding partner may be considered to be the binder of the ligand. An example of such a binding partner is avidin in the specific ligand-binder pair comprising a biotin-avidin complex.

Where, for example, a binder for an auxiliary ligand is a species which has a part which is capable of undergoing a specific binding reaction with the auxiliary ligand and a part which provides a primary species (said primary species being a binder for a primary species) which is capable of undergoing a specific binding reaction with a primary species it will be understood that the part capable of undergoing specific binding with the auxiliary ligand may be regarded as an "auxiliary function". An example of a species which has a part capable of undergoing a specific binding reaction with an auxiliary ligand and a part which is a primary species capable of undergoing a specific binding reaction with a primary species is a bifunctional antibody.

In view of the foregoing disclosure, it will be appreciated that a bifunctional antibody may have a part which provides a primary function and a part which

provides an auxiliary function. Also it will be appreciated that, if desired, a bifunctional antibody may be, for example, such as to have parts which provide different auxiliary functions.

Examples of support materials which may find application in accordance with the present invention are solid phase materials such as a reaction vessel wall, insoluble polysaccharides, microparticles (e.g. particulate microcellulose), polystyrene (e.g. in the form of wells, beads, microtitre plates, discs, sticks or tubes), cross-linked dextran (e.g. Sephadex), insoluble polymer structures, glass surfaces, derivatised silica surfaces (e.g. having silyl groups with chemical functions attached), soluble polymers attached to a suitable surface (e.g. a glass surface), microparticulate materials with entrapped ferrous oxide (magnetisable particles), nylon and polyamides.

By way of further example, a support material may be in the form of a carrier (e.g. a tape) which may be moved from a sample application means, for applying a sample to the carrier, to a detection means thereby to allow successive (e.g. continuous) detection for a plurality of different entities; thus, for example, successive (e.g. continuous) multi-entity detection (e.g. multi-analyte species detection) may be effected.

It will be appreciated that some types of support materials may be inappropriate for use with some entities to be detected. Thus, for example, where the entity to be detected is a metal ion, or contains a metal, the use of some types of support materials may be inappropriate (e.g. support materials containing entrapped iron oxide may give rise to unacceptable interference).

Also, it is to be understood that some types of support materials may not be appropriate for some types of detection in accordance with the present invention. Thus, for example, particulate support materials may not be appropriate where a plurality of zones is required on

a support material and said zones are respectively to provide an auxiliary ligand, and/or an auxiliary binder and an additional immunological species.

An auxiliary species (e.g. auxiliary ligand or an auxiliary binder) may be, for example, provided on a support material in any suitable manner, and it is to be understood that in this Specification "providing on a support material" and "provided on a support material" embrace, for example, situations where the support material carries an auxiliary species (e.g. an auxiliary ligand or an auxiliary binder), and situations where the support material itself, or a part of the support material itself, provides an auxiliary species (e.g. an auxiliary ligand).

Thus, for example, an auxiliary species, being an auxiliary ligand, may be provided by chemical groups or units of the support material. Alternatively, an auxiliary species (e.g. auxiliary ligand or an auxiliary binder) may be, for example, attached to the support material in any suitable manner (e.g. by covalent linkage or adsorption). Where the support material is, for example, a polymer, units of the polymer may act as an auxiliary ligand. Also, by way of example, surface groups present on a support material, such as polystyrene or modified silica, may act as an auxiliary ligand.

By way of example, the support material may, if desired, provide oligomers or polymers of an auxiliary ligand.

Where an auxiliary species (e.g. auxiliary ligand or an auxiliary binder) is attached to a support material the auxiliary species (e.g. auxiliary ligand or auxiliary binder) may be directly attached to the support material or indirectly attached to the support material via other species (e.g. a carrier protein).

In one embodiment the present invention provides a method which also includes the step of attaching, either

directly, or indirectly, an auxiliary species to a support material.

By way of further example, the surface of a support material may be activated thereby to permit attachment of an auxiliary species (e.g. auxiliary ligand or auxiliary binder); for example, the surface of a suitable support material may be activated by chemical treatment to provide free amino groups to which an auxiliary ligand or an auxiliary binder may be linked.

Further, by way of example, an oligomer or oligomers of an auxiliary ligand, or an oligomer or oligomers of an auxiliary binder, or a polymer or polymers of an auxiliary ligand, or a polymer or polymers of an auxiliary binder may be attached directly or indirectly to a support material.

Thus for example, oligomers of an auxiliary ligand or oligomers of an auxiliary binder or polymers of an auxiliary ligand or polymers of an auxiliary binder may be attached to free amino groups on a support material.

Also, by way of example, an auxiliary ligand or an auxiliary binder may be linked (e.g. covalently or otherwise) to a further species (e.g. a carrier protein or a polymer) and the further species may be associated with the support material such that the auxiliary ligand or auxiliary binder may become indirectly provided on the support material.

By way of further example, it is possible to link (e.g. covalently or otherwise) oligomers or polymers of auxiliary ligand or auxiliary binder to a further species (e.g. a carrier protein or a polymer) and the further species may be associated with the support material such that oligomers of auxiliary ligand or auxiliary binder or polymers of auxiliary ligand or auxiliary binder may become indirectly provided on a support material.

An additional immunological species may be, for example, provided on a support material in any suitable manner and it is to be understood that "providing on a

support material" and "provided on a support material" embrace, for example, situations where the support material carries an additional immunological species.

An additional immunological species (e.g. a binder or a ligand as is appropriate) may be attached to the support in any suitable manner (e.g. by covalent linkage or adsorption).

Where an additional immunological species is attached to a support material the additional immunological species may be directly attached to the support material or indirectly attached to the support material via other species (e.g. a carrier protein).

In an embodiment the present invention provides a method which also includes the step of attaching, either directly, or indirectly, an additional immunological species to a support material.

If appropriate, by way of example, polymers or oligomers of additional immunological species may be used in accordance with the present invention.

Where an auxiliary species or an additional immunological species is attached to a support material, attachment of the auxiliary species or additional immunological species may be effected at any desired time. By way of example, an auxiliary species may be allowed to bind with an appropriate auxiliary species reaction partner before, or after, the auxiliary species is attached to the support material; however, in general, it may be more convenient to attach an auxiliary species to the support material before bringing together the auxiliary species and the auxiliary species reaction partner.

It is to be understood that the use of oligomers or polymers of auxiliary species or additional immunological species may be advantageous in certain circumstances. Thus, for example, by use of an oligomer or a polymer of an auxiliary species or an additional immunological species more auxiliary species or more additional

immunological species may be provided (e.g. on a given surface area) than it is possible to provide when using only single "units" of auxiliary species or additional immunological species. Accordingly, for example, the provision of more auxiliary species or more additional immunological species offers the possibility of faster reactions since more auxiliary species or more additional immunological species is available to undergo reaction.

Where, for example, a primary species is to become associated with a support material by use of an auxiliary species or an additional immunological species this may be effected in any suitable manner and at any suitable time.

Thus, for example, a primary species may be associated with an auxiliary species reaction partner either before, or after, binding between the auxiliary species reaction partner and the auxiliary species has taken place.

Alternatively, by way of example, an auxiliary ligand of a selected auxiliary ligand-auxiliary binder system may be associated with a primary species before, or after, binding between the auxiliary ligand and the auxiliary binder of the system and before, or after, binding between the additional immunological species and the auxiliary binder of the system.

Where, for example, an additional immunological species is a primary antibody, binding between the additional immunological species reaction partner and the primary antibody may take place before, or after, binding of the additional immunological species reaction partner with another primary species.

Reaction between an additional immunological species and an additional immunological reaction partner may be effected at any suitable time.

It is to be understood that an auxiliary species reaction partner, or an auxiliary ligand or binder of a selected auxiliary ligand-binder system (e.g. of an

auxiliary ligand-auxiliary binder system), may be associated with a primary species by any suitable linkage; such a linkage may include a non-specific binding link or links (e.g. a covalent link or links or adsorption) or a specific binding link or links, or any combination of such links.

By way of example, an auxiliary species reaction partner, or a binder (e.g. an auxiliary binder) or an auxiliary ligand of a selected auxiliary ligand-binder system, may be arranged to be linked, either directly or indirectly, with a primary species by means of any suitable linkage. An example of such a linkage is one which involves a link, of a non-specific binding type, to the auxiliary species reaction partner or to the auxiliary ligand.

An example of a link of a non-specific binding type is a covalent link. Thus, for example, the linkage may involve a covalent link to an auxiliary species reaction partner, or to an auxiliary ligand of a selected auxiliary ligand-binder system.

Another example of a link of a non-specific binding type is a link which involves adsorption. Thus, for example, an auxiliary species reaction partner, or an auxiliary ligand, or a binder, and a primary species may be linked by being adsorbed on a suitable material (e.g. a carrier material such as latex particles).

As hereinbefore disclosed an auxiliary species reaction partner, or a binder or an auxiliary ligand, of a selected auxiliary ligand-binder system, may be linked with a primary species directly or indirectly (e.g. via other species).

Thus, for example, where an auxiliary species reaction partner, or a binder, or an auxiliary ligand (of a selected auxiliary ligand-binder system) is arranged to be linked directly to a primary species the linkage may be a link of a non-specific binding type (e.g. a covalent link) between the auxiliary species reaction partner, or

the binder, or the auxiliary ligand, and the primary species.

By way of further example, where an auxiliary species reaction partner, or a binder or an auxiliary ligand (of a selected auxiliary ligand-binder system), is arranged to be linked indirectly to a primary species the linkage may include one or more other species, and one or more links, as required, one of the links being a link, of a non-specific binding type, to the auxiliary species reaction partner, or to the binder, or to the auxiliary ligand.

It is to be understood that where, for example, one or more links are involved in the linkage, and one of the links is a link of a non-specific binding type to the auxiliary species reaction partner, or to the binder, or to the auxiliary ligand (of a selected auxiliary ligand-binder system), any further link or links in the linkage may be of any suitable type (e.g. non-specific binding type or specific binding type); thus, for example, the linkage may include a further link or links of a non-specific binding type, or may include a further link or links of a specific binding type (e.g. ligand-binder type), or a mixture of types of links (e.g. non-specific binding type and specific binding type).

Where an auxiliary species reaction partner, or a binder or an auxiliary ligand (of a selected auxiliary ligand-binder system) is linked to a primary species via one or more other species, said other species or one of said other species may be, for example, a second antibody or a ligand or a binder and, for example, the auxiliary species reaction partner, or the binder or auxiliary ligand, may be covalently linked to said other species or said one of said other species.

By way of example, more than one auxiliary ligand-binder systems may be used in a link by means of which a primary species may become associated with a support material. For example, an auxiliary species reaction

partner may be arranged to be associated with a primary species via a link which includes one or more auxiliary ligand-binder systems.

A primary species may be, for example, a primary antibody or a ligand (e.g. an antigen). It is to be understood that, for example, a primary species may be an antibody to an entity to be detected or an antibody to an authentic entity to be detected; it will be appreciated that, for a given immunoassay, the antibody to the entity to be detected and the antibody to the authentic entity to be detected will be the same antibody. It is also to be understood that a primary species may be, for example an entity to be detected or an authentic entity to be detected.

The present invention may find application in any suitable form of immunological detection or immunoassay examples of which are heterogeneous immunoassay methods, competitive immunoassay methods, homogeneous immunoassay methods, non-competitive immunoassay methods, sandwich immunoassay methods and direct immunoassay methods.

The present invention may find application in, for example, label-free or detectable species-dependent (e.g. tracer species-dependent) assay methods such as enzyme-immunoassay, fluoro-immunoassay and radio-immunoassay. By way of example, the present invention may find application in antibody-labelled or antigen-labelled assays.

By way of example, in accordance with an embodiment of the present invention, a plurality of different regions or zones of a support material may be arranged such that a selected specific separation may be effected at a given region or zone.

Thus, by way of example, one region or zone of a support material may be arranged to provide an auxiliary species (e.g. an auxiliary ligand, or an auxiliary binder), and another region or zone may be arranged to provide an additional immunological species, such that by

use, respectively, of an appropriate auxiliary species reaction partner or an appropriate additional immunological species reaction partner, different specific separations may be effected on different regions or zones of the support material.

From the foregoing disclosure, it will be appreciated that in accordance with the present invention, any suitable number of different auxiliary species and any suitable number of different additional immunological species may be provided on a support material; such auxiliary species or additional immunological species may be provided in selected regions or zones of a support material as is desired.

It will be appreciated that, where an auxiliary species being an auxiliary ligand is, or auxiliary species being auxiliary ligands are, provided on a support material and another auxiliary species being an auxiliary binder is, or auxiliary species being auxiliary binders are, also provided on a support material, the auxiliary ligand or ligands and the binder or binders may be chosen such that they are from different auxiliary ligand-binder pairs.

From the foregoing disclosure it will be appreciated that the present invention may be utilised, for example, to effect a first selected separation at a first region of a support material, a second selected separation at a second region of a support material, and so forth using as many regions as required to effect a desired number of separations. It is to be understood that the number of separations it is desired to effect may be determined by the number of entities for which it is desired to "probe" in a given application.

By way of example, a support material having a plurality of regions or zones for effecting a plurality of specific separations may be contacted with a reaction mixture in what may be termed a "simultaneous" mode of

operation (i.e. a reaction mixture may be exposed to a support material in one operation).

Thus, for example, in a "simultaneous" mode of operation in accordance with the present invention, one, or a plurality of, auxiliary species and one, or a plurality of, additional immunological species may be provided on a given support material.

Alternatively, by way of example, a reaction mixture may be contacted sequentially with a set of support materials each of which support materials is capable of effecting a specific separation which is different from that which may be effected by another member of the set. Thus, upon sequential contacting of a reaction mixture with members of the set, different entities (if present in a sample) will appear on different support materials.

Thus, members of the set may act as "probes" for particular entities.

Accordingly, for example, a "sequential" mode of operation may be utilised in accordance with the present invention.

By way of example, where a set of support materials is used to effect a "sequential" mode of operation, any suitable selection of auxiliary species and additional immunological species may be utilised. Thus, for example, one member of a set may provide one auxiliary species and another member of a set may provide an additional immunological species. A set may have as many members as is required to provide a desired number of different auxiliary species and different additional immunological species.

By way of further example, rather than using a set of separate support materials, a single support material may provide a plurality of regions to which a reaction mixture may be introduced sequentially (e.g. by use of a support material in the form of a wick, or similar arrangement, having regions that a reaction mixture meets sequentially as it travels along the support material).

For example, one region may provide an auxiliary species and another region may provide an additional immunological species; it will be appreciated that as many regions as is desired, and as is practically useable, may be employed to provide a desired number of different auxiliary species and different additional immunological species.

By way of example, where a support material is in a form which permits, and the detectable species is of a suitable type, detectable species signal from each of a plurality of support material structures may be measured separately (e.g. in separate tubes).

Thus, by way of example, where the form of a support material permits and the detectable species is, for example, an enzyme, enzyme activity on a support material may be measured separately. Thus, for example, where the support material is in a suitable form (e.g. dip-sticks), enzyme activity on each of a plurality of support material structures may be measured separately in separate tubes containing appropriate enzyme substrates. The amount of product formed in each tube may be measured separately by any suitable means (e.g. spectrophotometry).

According to a further aspect of the present invention there is provided a sequential separation method, suitable for use in an immunological method for the detection of a plurality of entities, which sequential separation method includes the use of one, or a plurality of, auxiliary species and one, or a plurality of, additional immunological species.

According to a further aspect of the present invention there is provided a method, suitable for use in immunological detection of a plurality of entities, which method includes the use of a sequential separation method in accordance with the present invention.

Entities to be detected may, for example, be detected by any suitable means, such as those known in

the art, whilst still associated with a support material; alternatively, by way of example, entities to be detected may be eluted from a support (e.g. using suitable buffer solutions) and then subjected to detection.

The present invention may find application in any suitable assay configuration. Thus, for example, the present invention may find application in:

(a) competitive binding assays (either ligand-labelled or antibody labelled), and

(b) non-competitive sandwich assays.

By way of example, when utilising the present invention with any of the assay types immediately hereinbefore disclosed in (a) or (b) any suitable combination of auxiliary species and additional immunological species may be provided on a support material.

Thus, the present invention provides, for example, immunological detection or immunoassay which includes a separation method in accordance with the present invention.

By way of example, any suitable detectable species may be used in accordance with the present invention in the detection of an entity to be detected. It is to be understood that a detectable species may be, for example, a detectable structure.

Examples of detectable species are enzymes (e.g. alkaline phosphatase, β-galactosidase and horse-radish peroxidase), species capable of giving a fluorescent signal (e.g. fluorophores (or polymeric fluorophores)), chemiluminescent compounds, bioluminescent compounds, radioisotopes, dyes, ligand species (or polymers of a ligand species), and binder species (or polymers of binder species. Examples of fluorophores are fluoresceins, coumarins and rhodamine.

By way of example, an enzyme may be detected by a corresponding substrate, fluorophores and radioisotopes may be detected directly with suitable detectors, ligand

species may be detected by use of binder species therefor, said binder species being associated with tracer species, binder species may be detected by use of ligand species therefor, said ligand species being associated with tracer species.

The tracer species may be, for example, any suitable tracer species such as those known in the art relating to protein binding assays (e.g. immunoassays). (It is to be understood that a tracer species may also be considered to be a signal species or a labelling species.)

Examples of tracer species are enzymes (e.g. alkaline phosphatase, β-galactosidase and horse-radish peroxidase), species capable of giving a fluorescent signal (e.g. fluorophores (e.g. fluoresceins, coumarins or rhodamine)), chemiluminescent compounds, bioluminescent compounds, radioisotopes and dyes.

Detection or measurement of a signal from a detectable species may be carried out in any suitable manner such as those known in the immunochemical field.

Where, for example, a ligand species or a binder species is used (as hereinbefore disclosed) as a detectable species any suitable ligand species or binder species may be utilised.

By way of further example, a detectable species comprising an enzyme may be detected by means of an enzyme induced change.

The enzyme induced change may be, for example, a colour change, or a fluorescence change, or a luminescence change, or an electro-chemical change.

Where a detectable species is used in accordance with the present invention, the detectable species may be, for example, arranged to be associated, in any suitable manner and at any suitable time, with any chosen species or entity; thus, for example, a detectable species may be associated (as appropriate to an immunoassay being utilised) with an authentic entity

(being an authentic entity to be detected) or with a primary antibody, or with a second antibody.

It is to be understood that a separation method in accordance with the present invention may be used, for example, in the separation of antibody bound and unbound fractions of a detectable species in an immunoassay method.

Entities to be detected may be, for example, any suitable entities capable of undergoing a specific binding reaction.

Thus, for example, entities to be detected may themselves be analyte species. For example, entities to be detected may be analyte species comprising ligands (e.g. antigenic ligands such as haptens). By way of further example, entities to be detected may be analyte species comprising antibodies.

Examples of entities which are analyte species which may be detected in accordance with the present invention are:

(a) steroid hormones such as progesterone, 17α-hydroxy progesterone or estradiol (e.g. in a sample of blood, serum, saliva, urine or milk),

(b) hormones such as thyroid hormone (e.g. thyroxine or triiodothyronine),

(c) steroids in extracts (e.g. extracts of solids or liquids),

(d) drugs such as drugs of abuse (e.g. phenobarbital) and therapeutic drugs (e.g. digoxin) (in for example, a sample of blood, serum, saliva or urine),

(e) polypeptide hormones (e.g. hCG) in, for example, a sample of blood or urine,

(f) tumour markers such as marker proteins (e.g. in a sample of blood or serum),

(g) protein antigens,

(h) blood proteins (e.g. human serum albumin, immunoglobulins (e.g. IgG), enzyme markers or receptors),

(i) marker proteins in urine resulting from kidney diseases,

(j) pesticides such as insecticides, or herbicides (e.g. in water or soil),

(k) toxins (such as those extracted from feeds and food stuffs),

(l) micro-organisms (e.g. viruses and bacteria), and

(m) antibodies to micro-organisms.

Further examples of entities which are analyte species which may be detected in accordance with the present invention are complexes of metals.

Thus, the present invention may find application in, for example, the detection of complexes of metals such as strong metal complexes which may be regarded as toxic (e.g. in biological terms when present in the environment).

An example of a metal complex which may be detected in accordance with the present invention is methyl mercury.

By way of further example, entities to be detected may be entities which carry or include analyte species. Thus, for example, entities to be detected may be species formed by interaction of analyte species with a suitable agent or agents.

For example, where analyte species are metal ions the agent or agents may be a complexing agent or complexing agents capable of interacting with the metal ions to form entities (e.g. metal ion complexes) for detection. Thus, for example, metal ions may be formed into complexes of metals by use of a complexing agent or complexing agents and the complexes thus formed may act as entities to be detected in accordance with the present invention.

Thus, for example, metals may be detected in accordance with the present invention.

Examples of complexing agents are chelating agents.

Examples of chelating agents suitable for forming metal chelates are:

ethylene diamine tetra acetate (EDTA), diethylene triamine penta acetate (DTPA), cyclohexylene-dinitrilo tetra acetic acid (CDTA), 1-benzyl-EDTA, derivatives of 1-benzyl-EDTA, 8-hydroxyquinoline, derivatives of 8-hydroxyquinoline, and deferoxamine.

The following are examples of metals which may be analyte species capable of being formed into entities to be detected comprising metal complexes:

calcium (Ca^{II}), iron (Fe^{II}, Fe^{III}), cobalt (Co^{II}, Co^{III}), aluminium (Al^{III}), zinc (Zn^{II}), lead (Pb^{II}), copper (Cu^{II}, Cu^{I}), cadmium (Cd^{II}), vanadium (V^{II}, V^{III}), silver (Ag^{I}, Ag^{II}), mercury (Hg^{I}, Hg^{II}, indium (In^{III}), manganese (Mn^{II}) and nickel (Ni^{II}).

From the foregoing disclosure, it will be appreciated that in an embodiment of the present invention a method may include the step of forming a complex of an analyte species, said complex thus formed providing an entity to be detected.

Also from the foregoing disclosure, it will be appreciated that in an embodiment of the present invention a method may include the step of linking, either directly or indirectly, an agent to a binding species.

The following are examples of applications in which the present invention may be used in respect of multi-entity detection:

(1) fertility status (male or female) measurements,
(2) fetal health indicators profile measurements,
(3) newborn health status measurements,
(4) thyroid function testing,
(5) high risk infectious microbial disease screening,
(6) autoantibodies detection,
(7) tumour marker detection,
(8) kidney damage marker detection,
(9) screening for drugs of abuse,

(10) screening for pesticides and residues thereof.

The present invention may find application, for example, in the detection of entities to be detected, in any suitable sample. Thus, for example, samples of water, soil, living species (such as plants (e.g. vegetables) or animals) or air may provide entities to be detected, for detection in accordance with the present invention. By way of example, air-borne species may be entities to be detected and such air-borne species may be, for example, extracted from a sample of air and then subjected to detection. Examples of biological samples in which entities to be detected, may be detected in accordance with the present invention are blood, plasma, serum, urine, saliva and milk. Entities to be detected may be, for example, present in water, an aqueous preparation or a fluid extract (e.g. one prepared by solvent extraction). Entities to be detected may be, for example, haptens.

In accordance with the present invention, by way of example, it is possible to use a support material having a chosen combination of auxiliary species and additional immunological species, each of which is provided at a different region of the support material for the detection of different groups of entities.

By way of example, the same support material, having a given combination of auxiliary species and additional immunological species, may be used (in conjunction with, respectively, corresponding reaction partners), to effect separation of a number of different groups of entities.

This is so since the same combination of auxiliary species and additional immunological species may be used to effect selective separation whatever the entities.

This is possible since selectiveness with respect to which entities or groups of entities are to be detected in a given assay may be determined, for example, by the choice of primary species with which a particular

auxiliary species reaction partner or an additional immunological species reaction partner may be associated.

It is to be understood that by use of a suitable combination of auxiliary species and additional immunological species, different entities or different entities associated with detectable species may be selectively separated at different regions of the support material; these regions may then be interrogated separately in a manner which is appropriate to the type of detectable species employed (e.g. optical measurements in the case of a fluorophore) to facilitate detection of a plurality of different entities (each at a different selected region of the support material).

It will be appreciated that, for example, a serum protein blocking reagent may, optionally, be used in accordance with the present invention if required for a particular application.

It will also be appreciated that, for example, there may be a need to dilute samples (e.g. with suitable buffer solutions) prior to subjecting to detection.

It is to be understood that the present invention may find application in qualitative detection of an entity or entities, which may also be considered to be qualitative analysis, or in quantitative detection of an entity or entities, which may also be considered to be quantitative analysis (i.e. measurement or determination of an entity or entities).

Also, it will be appreciated that, for example, the present invention may be utilised with samples which contain one or more entities to be detected and with samples which contain substantially no entities to be detected (e.g. such as in the case of a "zero" standard sample or an "unknown" sample, which upon subjecting to detection in accordance with the present invention, is found to contain substantially no entities to be detected). Thus, it will be appreciated that the present invention may be used to "probe" for a plurality of

entities, whether or not a particular entity is, or entities are, present in a particular sample.

It will also be appreciated that the present invention may be applied to the detection of a number of different entities over differing concentration ranges.

Further it will be appreciated, by way of example, that by use of standard quantities of authentic entities to be detected, calibration may be effected such that, subsequently, unknown quantities of entities to be detected may be determined.

From the foregoing disclosure it will be appreciated that, for example, where a primary species is associated with an auxiliary species reaction partner and the auxiliary species reaction partner undergoes binding with auxiliary species provided on a support material to effect a separation in accordance with the present invention, the primary species will become indirectly linked to the support material; it will also be appreciated that when such a separation is effected any other species associated with the primary species (e.g. another primary species or a detectable species) will become indirectly linked to the support material.

Also, from the foregoing disclosure it will be appreciated that where, for example, a primary species is associated either directly or indirectly with an additional immunological species reaction partner and the additional immunological species reaction partner undergoes binding with an additional immunological species, the primary species will become indirectly linked to the support material; it will be appreciated that when such a separation is effected any other species associated with the primary species (e.g. another primary species or a detectable species) will become indirectly linked to the support material.

Thus, by use of a combination of auxiliary species, and additional immunological species and a plurality of different primary species (associated respectively with

an appropriate auxiliary species reaction partner or partners and an appropriate additional immunological species reaction partner or partners it is possible to effect a plurality of specific separations and thus possible to carry out a plurality of detections for a plurality of entities to be detected.

It is to be understood that different auxiliary species-auxiliary species reaction partner pairs and different additional immunological species-additional immunological reaction partner pairs may be chosen such that there is no unacceptable interference between any of the pairs. Thus, for example, such pairs may be chosen such that there is no degree of interference such as to cause unacceptable interference with the capability of effecting a plurality of specific separations.

It is to be understood that the present invention may, for example, facilitate the use of only one procedure or one set of procedures in the testing of a sample for a plurality of entities.

Thus, for example, in one embodiment of the present invention it is not necessary to perform an assay procedure or a set of assay procedures separately for each entity in a sample. Thus, for example, the present invention may be used in what may be considered to be "simultaneous" detection of a plurality of entities; it will be appreciated that "simultaneous" relates to the performance of a procedure or a set of procedures and not necessarily to various reactions which may occur in a reaction mixture at different speeds and times.

It will be appreciated that antibodies suitable for use in accordance with the present invention may be prepared by any suitable method, for example those known for the raising of polyclonal or monoclonal antibodies; thus, antibodies may be raised, for example, by immunising animals with conjugates made of suitable substances; the product obtained by immunising animals

may be purified as desired (e.g. by the use of affinity chromatography) to obtain the required antibodies.

The term "antibody" as used in this Specification embraces whole antibody or antibody fragments such as Fab and $(Fab)_2$ and, accordingly, the term "antibodies" used herein embraces whole antibodies and antibody fragments.

Any suitable configuration may be utilised in accordance with the present invention when carrying out an assay.

For example an auxiliary species may be used in a competitive assay method or a non-competitive assay method and an additional immunological species may be used in a competitive assay method or a non-competitive assay method.

By way of example, by suitable choice of configuration and numbers of different auxiliary species and additional immunological species, it may be possible to effect a number of different competitive assays, a number of non-competitive assays and non-competitive assays.

Where, for example, it is desired to use an auxiliary species in a competitive assay method, an auxiliary species may be introduced in high concentration to support material or a support material itself may be arranged to provide an auxiliary species.

Also, an auxiliary species reaction partner may be linked (e.g. by covalent linking), to an authentic entity of interest. An antibody to the entity to be detected may also be labelled with an appropriate detectable species capable of giving rise to a signal (e.g. a fluorescent substance or an enzyme). It will be appreciated that the antibody to the entity to be detected may be considered to be a primary antibody.

The primary immune reaction may be expected to attain or approach equilibrium in a short period of time (e.g. at a temperature of 37°C to 42°C) thereby to produce a primary immune reaction mixture. It will be

appreciated that the primary reaction mixture may contain, *inter alia*, bound and unbound fractions.

Subsequently the primary immune reaction mixture is brought into contact with an "excess" of the auxiliary species provided on the support material.

(It will be appreciated that "excess" as used in the immediately preceding paragraph means an "excess" in terms of auxiliary species sites with respect to the corresponding auxiliary species reaction partner.)

Because the auxiliary species is present in very large excess with respect to the corresponding auxiliary species reaction partner, binding reaction between the two may be expected to occur rapidly and efficiently with a high proportion of the auxiliary species reaction partner becoming bound to the support material. By way of example, in some circumstances, but not all, as much as 99% of the auxiliary species reaction partner may become bound to the support material.

Thus, by means of binding between the auxiliary species and the auxiliary species reaction partner, some of the primary antibody carrying the detectable species may be retained on the support material by virtue of being linked to appropriate authentic entities which, in turn, are linked to the auxiliary species reaction partner. The proportions of retained primary antibody carrying detectable species will depend upon the concentration of "competing" entity, if any, in a given sample; the competing entity may be a standard or an unknown quantity.

Washing of the support material with appropriate buffers may be used to remove unbound materials and detectable species activity associated with selected regions of the support material may be measured by any suitable method such as those known in the art.

Optionally, detectable species activity may be eluted into solution using appropriate buffers to facilitate measurement of such species.

In some embodiments of the present invention (e.g. embodiments involving the use of an optical immunosensor based on the surface plasmon resonance principle or on evanescent wave fluorescence) a washing step may not be required; thus, detectable species activity associated with the support material (which may be, for example, a sensor surface) may be measured in the presence of unbound material.

It will be understood that, by use of standard quantities of authentic entities, calibration may be effected such that, subsequently, unknown quantities of entities may be determined. It will also be appreciated that, in a competitive immunoassay, as an amount of a given entity in a sample increases, the amount of authentic entity corresponding thereto retained on the support material decreases (and hence the amount of detectable species retained on the support material decreases); it will also be appreciated that, conversely, as the amount of a given entity in a sample decreases, the amount of authentic entity corresponding thereto retained on the support material increases (and hence the amount of detectable species retained on the support material increases), reaching a maximum when no entity is present in a sample.

It will be appreciated that in the competitive assay immediately hereinbefore disclosed, the auxiliary species may be, for example, an auxiliary ligand (in which case the corresponding auxiliary species reaction partner may be a binder). Where, for example, the ligand is an antigenic ligand (e.g. a hapten), the auxiliary species reaction partner may be, for example, an auxiliary binder which is an antibody (which may be considered to be an anti-auxiliary ligand antibody). Conversely, where the auxiliary species is an auxiliary binder (e.g. an antibody), the auxiliary species reaction partner may be an auxiliary ligand.

By way of example, where it is desired to use an auxiliary species in a non-competitive immunoassay method which involves a separation method in accordance with the present invention the following may be utilised:

(a) an antibody to an entity to be detected conjugated to a detectable species capable of giving rise to a signal (e.g. a fluorescent substance or an enzyme); this antibody may be considered to be a signal antibody;

(b) a hybrid complex comprising an "immobilising" antibody conjugated to an auxiliary species reaction partner; the immobilising antibody is an antibody to an entity to be detected;

(c) an entity to be detected (which may be, for example, an antigenic substances of high molecular weight);

(d) a support material which itself provides an auxiliary species (being a ligand) or has attached thereto an auxiliary species (e.g. an auxiliary ligand or an auxiliary binder).

Also, by way of example, in a non-competitive immunoassay method which includes a separation method in accordance with the present invention the following may take place:

(i) an entity of interest and the "signal" antibody conjugated to the detectable species are mixed in solution whereupon an entity present is bound by the signal antibody;

(ii) to the reaction mixture formed in (i) in solution a moderate "excess" of the hybrid complex is added whereupon an immune complex is formed in which the entity is "sandwiched";

(iii) the reaction mixture formed in (ii) is brought into contact with an "excess" of the auxiliary species provided on the support material such that binding of the auxiliary species reaction partner with the auxiliary species takes place. By virtue of this binding reaction, only

"signal" antibody bound to the entity to be detected becomes attached to the support material.

Washing the support material with appropriate buffer may be used to remove unattached "signal" antibody.

Detectable species left on the support material, the amount of which is directly proportional to the concentration of the entity, may be measured by any suitable method such as those known in the art.

Optionally, detectable species activity may be eluted into solution using appropriate buffers to facilitate measurement of such species.

In some embodiments of the present invention (e.g. embodiments involving the use of an optical immunosensor based on the surface plasmon resonance principle or on evanescent wave fluorescence) a washing step may not be required; thus, detectable species associated with the support material (which may be, for example, a sensor surface) may be measured in the presence of unbound material.

It will be understood that by use of standard quantities of authentic entity calibration may be effected such that, subsequently, unknown quantities of entity may be determined.

It will be appreciated that where the auxiliary species is a ligand the auxiliary species reaction partner may be a binder and where the auxiliary species is an auxiliary binder the auxiliary species reaction partner may be an auxiliary ligand.

Where an additional immunological species is utilised in a competitive assay method or a non-competitive assay method any suitable configuration may be used.

Thus, for example, where the additional immunological species is a primary species, a competitive or non-competitive immunoassay method may be carried out in accordance with known procedures.

By way of further example, where the additional immunological species is a second antibody, primary species may take part in a competitive or non-competitive assay, and the second antibody may be utilised to associate primary antibody, and any desired species associated therewith, with a support material.

Where the additional immunological species is an antibody capable of binding an auxiliary species comprising an auxiliary binder, a competitive method or non-competitive assay method may be carried out utilising, *inter alia*, an auxiliary species and an auxiliary species reaction partner in a manner similar to that hereinbefore disclosed, with the exception that the auxiliary species is not provided on a support material, and the additional immunological species may be used to associate a binder of an auxiliary ligand-binder system, and any desired species associated therewith, with a support material.

Where the additional immunological species is a binder capable of binding a complex which complex is a complex of an auxiliary species and an auxiliary species reaction partner, a competitive assay method or a non-competitive assay method may be carried out utilising, *inter alia*, an auxiliary species and an auxiliary species reaction partner, and the additional immunological species may be used to associate a complex (which complex is a complex of the auxiliary species and the auxiliary species reaction partner), and any desired species associated therewith, with a support material.

Where the additional immunological species is a binder capable of binding part of a bifunctional antibody, a competitive assay method, or a non-competitive assay method, may be carried out and binding of the binder with part of the bifunctional antibody may be used to associate the bifunctional antibody, and any desired species associated therewith, with a support material.

It is to be understood that the part of the bifunctional antibody which is bound by a binder therefor may be considered to be acting as a ligand for that binder.

Where the additional immunological species is a binder capable of binding a complex which includes a bifunctional antibody, a competitive assay method or a non-competitive assay method may be carried out and binding of the complex by the binder may be used to associate the complex and any desired species associated therewith, with a support material.

Examples of combinations of species which may be provided on a support material or support materials and used in a multi-entity detection in accordance with the present invention are:

(a) an auxiliary ligand, a primary antibody, a first auxiliary binder, and an antibody to a second auxiliary binder;

(b) an auxiliary binder, a primary species in the form of an antigen, and a second antibody;

(c) an auxiliary ligand, a hapten (e.g. analyte derivative) and a second antibody;

(d) a plurality of different auxiliary binders and a second antibody.

It will be appreciated that in heterogenous immunochemical analysis (immunoassay) there is a requirement for the separation of bound and unbound fractions of detectable species in order to permit assessment of the distribution of detectable species between bound and unbound states. It may be considered that this separation step in an immunoassay method is one of the most important features of such a method.

The practical importance of a separation step is based upon the fact that the efficiency of the step, the simplicity (or otherwise) thereof, and the speed thereof may influence the general properties of an assay method and may influence the performance of an assay method.

The present invention offers the possibility of providing a multiplicity of different separation "systems"; this may offer the advantage of affording a wide scope of application. Thus, for example, the invention may find application where it is required to assay different entities, which entities require different separation systems and/or different separation conditions. For example, the invention may find application in circumstances where entities to be detected are present in different concentration ranges and thus require different treatment to perform a satisfactory assay. Also, binding affinity of a given pair of primary species may differ considerably from binding affinity of another given pair of primary species, and this may require the use of different separation systems which the present invention may offer.

In accordance with the present invention, an auxiliary species or an additional immunological species may be, for example, immobilised on a surface of a sensor device (e.g. on a glass surface, on a quartz surface, or on the surface of an electrode).

For example, in the use of direct optical immunosensors no detectable species is used and the binding of entities to be detected and antibodies may give rise to signal generation to permit detection (e.g. by surface plasmon resonance).

By way of further example, in the use of a detectable species labelled antibodies immunosensor, signal generation may be detected by a suitable method appropriate to the nature of the signal generated by the detectable species (e.g. a means for measuring fluorescent emission may be used when a detectable species is a fluorescence producing species).

By way of example, if desired, the present invention may make use of an inactive species or a plurality of inactive species which may be activated as required to be utilised in accordance with the present invention.

Thus, for example, an inactive species or a plurality of inactive species may be such as to be reconstitutable to an active form.

An inactive form of a species may be prepared by drying and/or freeze-drying (lyophilising).

Thus, for example, any species or combination of species suitable for use in accordance with the present invention may be rendered inactive (e.g. by drying and/or freeze-drying (lyophilising) so as to produce a reconstitutable reagent system or reconstitutable reagent material.

For example, an auxiliary species, or an additional immunological species, or a plurality of any of these, optionally, in any suitable combination, may be prepared in a reconstitutable form (e.g. by freeze-drying) such that, upon reconstitution, multi-entity analysis may be effected.

Reconstitution may be effected in any suitable manner, for example, by addition of a reconstituting agent such as a suitable solvent (e.g. water), or a suitable solution (e.g. an aqueous solution) or a suitable sample (e.g. an aqueous sample).

The present invention will now be further described, by way of example only, with reference to the accompanying Drawings in which:

Figure 1 is a diagrammatic representation of an apparatus in accordance with the present invention;

Figure 2 is a diagrammatic representation of another apparatus in accordance with the present invention;

Figure 3 is a diagrammatic representation of a further apparatus in accordance with the present invention;

Figure 4 is a diagrammatic representation of yet a further apparatus in accordance with the present invention; and

Figure 5 is a diagrammatic representation of a sensor in accordance with the present invention.

Referring now to Figure 1 of the accompanying Drawings there is shown apparatus 1 having a support material 2 which support material 2 has regions 3, 4, 5, 6, 7 and 8.

In operation the regions 3, 4, 5, 6, 7 and 8 of support material 2 may be such that at least one region provides an auxiliary species (not shown) and such that at least one other region provides an additional immunological species (not shown). The other regions may, respectively, provide a different auxiliary species or a different additional immunological species. Thus, for example, one of the regions 3, 4, 5, 6, 7 or 8 may provide an auxiliary species, another of the regions may provide an additional immunological species, another of the regions may provide a different auxiliary species or a different additional immunological species and so forth. Accordingly, it may be arranged that none of the regions 3, 4, 5, 6, 7 and 8 provides the same auxiliary species or the same additional immunological species. Thus it may be arranged that any suitable combination of auxiliary species and additional immunological species may be provided.

Upon contacting the apparatus with a material containing reaction partners for the auxiliary species and for the additional immunological species provided by the regions 3, 4, 5, 6, 7 and 8 each of the reaction partners will bind with its corresponding species on the support material.

Thus a plurality of specific separations may be carried out, after which each region will have collected substantially only one type of reaction partner (e.g. one type of auxiliary species reaction partner or one type of additional immunological species reaction partner).

Any detectable species associated with each region may then be detected by appropriate interrogation of each region separately. It will be appreciated that the type of interrogation will depend upon the particular detectable species involved (e.g. fluorescence measurements may be made when the detectable species is a fluorophore).

Thus, a sample may be tested for a plurality of different entities (i.e. assays may be carried out for a plurality of different entities using the same support material, and if desired, in the same reaction vessel).

It will be appreciated that if a material does not contain a reaction partner for each type of auxiliary species or additional immunological species one or more regions may not collect a reaction partner.

Upon contacting the apparatus with a material which contains a plurality of different auxiliary species reaction partners or additional immunological species reaction partners corresponding to auxiliary species or additional immunological species provided in the regions, each of the reaction partners in the material will collect at the region which provides its corresponding auxiliary species or additional immunological species as is appropriate.

Referring now to Figure 2 of the accompanying Drawings there is shown apparatus 10 having a support material 11, of substantially rectangular plate configuration, having areas 12 to 23 in which of each of areas 12 to 23 there is provided respectively regions 24 to 35.

In operation the regions 24 to 35 of support material 11 may be such that at least one of the regions provides an auxiliary species (not shown) and such that at least one other region provides an additional immunological species (not shown).

The other regions may provide different auxiliary species or additional immunological species in a manner

substantially similar to that as hereinbefore disclosed with respect to Figure 1 of the accompanying Drawings. Accordingly, it may be arranged that none of the regions 24 to 35 provides the same auxiliary species or the same additional immunological species.

Thus, it may be arranged that any suitable combination of auxiliary species and additional immunological species may be provided in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Also a plurality of specific separations may be carried out, and a sample may be tested for a plurality of entities (i.e. an assay may be carried out for a plurality of different entities) in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

The support material 11 may be identified in some convenient manner to facilitate the identification of the regions 24 to 35. Thus for example a small portion (e.g. a corner) of the support material 11 may be removed prior to use. For example, a small triangular portion of the outer corner of area 12 may be removed (as indicated by dotted line 36).

The apparatus 10 may be used, for example, in a horizontal position.

It will be appreciated that if a material does not contain a reaction partner for each type of auxiliary species or additional immunological species one or more regions may not collect a reaction partner.

Referring now to Figure 3 of the accompanying Drawings there is shown an apparatus 40. The apparatus 40 has a support material 41 in the form of a regular octahedral prism and thus it will be appreciated that Figure 3 is a plan view of such a prism.

The support material 41 has sides 42 to 49.

In operation regions of the sides 42 to 49 of the support material 41 may each have a region which provides

an auxiliary species or an additional immunological species.

Thus, at least one of the sides may have a region which provides an auxiliary species and at least one other side has a region which provides an additional immunological species. The other sides may have a region which may provide different auxiliary species or different additional immunological species in a manner substantially similar to that hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings. Accordingly, it may be arranged that none of the sides 42 to 49 provides the same auxiliary species or the same additional immunological species.

Thus, it may be arranged that any suitable combination of auxiliary species and additional immunological species may be provided in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Also a plurality of specific separations may be carried out and a sample may be tested for a plurality of different entities (i.e. assays may be carried out for a plurality of different entities) in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

The apparatus 40 may be used, for example, in a vertical position (i.e. with the sides 42 to 49 in a vertical position).

It will be appreciated that if a material does not contain a reaction partner for each type of auxiliary species or additional immunological species one or more regions may not collect a reaction partner.

Referring now to Figure 4 of the accompanying Drawings there is shown an apparatus 60 which has a support material 61 in the form of an absorbent wick (e.g. of cellulose paper). The support material 61 has regions 62 to 67.

Also the apparatus 60 has a supply reservoir 68, and a receiving reservoir 69.

In operation the regions 62 to 67 of the support material 61 may be such that at least one region provides an auxiliary species (not shown) and such that at least one other region provides an additional immunological species (not shown).

Regions 70 to 76 of the support material 61 are such as to provide no auxiliary species and no additional immunological species.

It may be arranged that each of the regions 62 to 67 provides different auxiliary species and different additional immunological species. Thus, the regions may provide different auxiliary species or additional immunological species in a manner substantially similar to that hereinbefore disclosed with respect to Figure 1 of the accompanying Drawings. Accordingly, it may be arranged that none of the regions 62 to 67 provides the same auxiliary species or same additional immunological species.

Thus, it may be arranged that any suitable combination of auxiliary species and additional immunological species may be provided in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Also a plurality of specific separations may be carried out, and a sample may be tested for a plurality of entities (i.e. assays may be carried out for a plurality of different entities) in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Thus, for example, a material (represented as 77) containing reaction partners for the auxiliary species and for the additional immunological species provided by the regions 62 to 67 may be provided in supply reservoir 68 and the support material may be dipped into the material 77.

As the material 77 rises up the support material 61, by means of capillary action, from supply reservoir 68 to receiving reservoir 69 reaction partner carried by the material will be retained at a different one of the regions 62 to 67 (i.e. a reaction partner will be retained at a region which provides its corresponding species on the support material).

Each of the regions 62 to 67 may be interrogated (by a method appropriate to any detectable species present) to detect the presence, absence or amount of different entities.

It will be appreciated that if a material does not contain a reaction partner for each type of auxiliary species or additional immunological species one or more regions may not collect a reaction partner.

It will be appreciated that the apparatus 60 may permit sequential separation in accordance with the present invention.

Referring now to Figure 5 of the accompanying Drawings, there is shown apparatus 80 having a member 81 from which depends a plurality of probes 82, 83, 84, 85 and 86.

(Although only 5 probes are shown, by way of example, the member 81 may carry as many probes as is desired.)

The probes 82 to 86 each comprise support material such that in operation the probes 82 to 86 may each provide a region each of which regions may provide an auxiliary species (not shown) or an additional immunological species.

Thus it may be arranged that at least one of the regions provides an auxiliary species and that at least one other of the regions provides an additional immunological species.

The other regions may provide different auxiliary species or different additional immunological species in a manner substantially similar to that as hereinbefore

disclosed with respect to Figure 1 of the accompanying Drawings. Accordingly, it may be arranged that none of the probes 82 to 86 provide a region which provides the same auxiliary species or same additional immunological species.

Thus, it may be arranged that any suitable combination of auxiliary species and immunological species may be provided in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Also, a plurality of specific separations may be carried out and a sample may be tested for a plurality of different entities (i.e. assays may be carried out for a plurality of different entities) in a manner substantially similar to that as hereinbefore disclosed with reference to Figure 1 of the accompanying Drawings.

Thus, for example, a material containing reaction partners for the auxiliary species and for the additional immunological species provided by the regions may be contacted with the apparatus 80 such that the material comes into contact with the probes 82 to 86.

Each reaction partner in the material will be retained on one of the probes 82 to 86 which provides its corresponding species.

Subsequently the probes may be individually interrogated for the presence of any detectable species thereon in any suitable manner. Thus, for example, where a detectable species is such as to give rise to an optical signal (e.g. fluorescence) then a probe may be connected to a suitable detector by an optical fibre (not shown) which is connected through the member 81 to an appropriate probe.

By way of further example, where a detectable species is such as to give rise to an electrical signal (e.g. an enzyme capable of generating an electrochemical signal) a probe may be connected to a suitable detector

by means of conductors (not shown) which are connected through the member 81 to an appropriate probe.

It will be appreciated that if a material does not contain a reaction partner for each type of auxiliary species or additional immunological species one or more regions may not collect a reaction partner.

By way of example, where a detectable species is an enzyme, enzyme activity on each of probes 82 to 86 may be measured separately in separate tubes each containing an appropriate enzyme substrate. The amount of product formed in each tube may then be measured separately by any suitable means (e.g. by spectrophotometry).

By way of example, it will be appreciated that regions to which reference is made in relation to Figures 1 to 5 of the accompanying Drawings may provide an auxiliary species and an additional immunological species in any suitable way. Thus, for example, a suport material may carry an auxiliary species, thus an auxiliary species may be attached to a support material, or a support material itself or a part thereof may provide an auxiliary species and a support material may carry an additional immunological species, thus an additional immunological species may be attached to a support material.

Claims

1. A separation method suitable for use in an immunological method for the detection of a plurality of entities, which separation method includes the use of an auxiliary species provided on a support material and the use of an additional immunological species (as hereinbefore defined) provided on a support material.

2. A method, suitable for use in immunological detection of a plurality of entities, which method includes the use of an auxiliary species provided on a support material and the use of an additional immunological species (as hereinbefore defined) provided on a support material.

3. A method as claimed in Claim 1 or Claim 2 wherein the auxiliary species is an auxiliary ligand or an auxiliary binder.

4. A method as claimed in any one of Claims 1 to 3 wherein the additional immunological species is a second antibody.

5. A method as claimed in any one of Claims 1 to 3 wherein the additional immunological species is a binder capable of binding with a binder of a selected auxiliary ligand-binder system.

6. A method as claimed in any one of Claims 1 to 3 wherein the additional immunological species is a primary species.

7. A method as claimed in any one of Claims 1 to 3 wherein the additional immunological species is a binder capable of binding with a complex which complex is a complex of an auxiliary species and an auxiliary species reaction partner.

8. A method as claimed in any one of Claims 1 to 3 wherein the additional immunological species is a binder capable of binding part of a bifunctional antibody or capable of binding a complex which includes a bifunctional antibody.

9. A method as claimed in any one of Claims 1 to 8 wherein an auxiliary species reaction partner is used and the auxiliary species reaction partner is an auxiliary binder, an auxiliary ligand, or a species which has a part which has an auxiliary function.

10. A method as claimed in any one of Claims 1 to 9 wherein there is used an auxiliary species and an additional immunological species, or a plurality of different auxiliary species and an additional immunological species, or an auxiliary species and a plurality of different additional immunological species, or a plurality of different auxiliary species and a plurality of different additional immunological species.

11. A method as claimed in any one of Claims 1 to 10 wherein an auxiliary species or an auxiliary species reaction partner is an antigenic ligand or non-antigenic ligand.

12. A method as claimed in any one of Claims 1 to 11 wherein a plurality of different regions or zones of a support material are arranged such that a selected specific separation may be effected at a given region or zone.

13. A method as claimed in any one of Claims 1 to 12 wherein a detectable species is used and the detectable species is an enzyme, a species capable of giving a fluorescent signal, a chemiluminescent compound, a bioluminescent compound, a radioisotope, a dye, a ligand species or a binder species.

14. A method as claimed in any one of Claims 1 to 13 wherein analyte species or entities to be detected are steroid hormones, thyroid hormones, steroids in extracts, drugs, polypeptide hormones, tumour markers, protein antigens, blood proteins, marker proteins, pesticides, toxins, micro-organisms, antibodies to micro-organisms, metal complexes or metal ions.

15. A method as claimed in any one of Claims 1 to 14 wherein the support material is a reaction vessel wall,

an insoluble polysaccharide, a microparticle, polystyrene, cross-linked dextran, an insoluble polymer structure, a glass surface, a derivatised silica surface, a magnetisable particle, nylon or a polyamide.

16. A method as claimed in any one of Claims 1 to 15 wherein a competitive immunoassay or a non-competitive immunoassay is effected.

17. A sensor, suitable for use in immunological detection of a plurality of entities, which sensor includes an auxiliary species and an additional immunological species (as hereinbefore defined), said auxiliary species and said additional immunological species being provided on a support material.

18. Apparatus, suitable for use in immunological detection of a plurality of entities, which apparatus includes an auxiliary species and an additional immunological species (as hereinbefore defined), said auxiliary species and said additional immunological species being provided on a support material or support materials.

19. A test-kit, suitable for use in immunological detection of a plurality of entities, which test-kit includes an auxiliary species and an additional immunological species (as hereinbefore defined), said auxiliary species and said additional immunological species being provided on a support material, or support materials.

Fig.1.

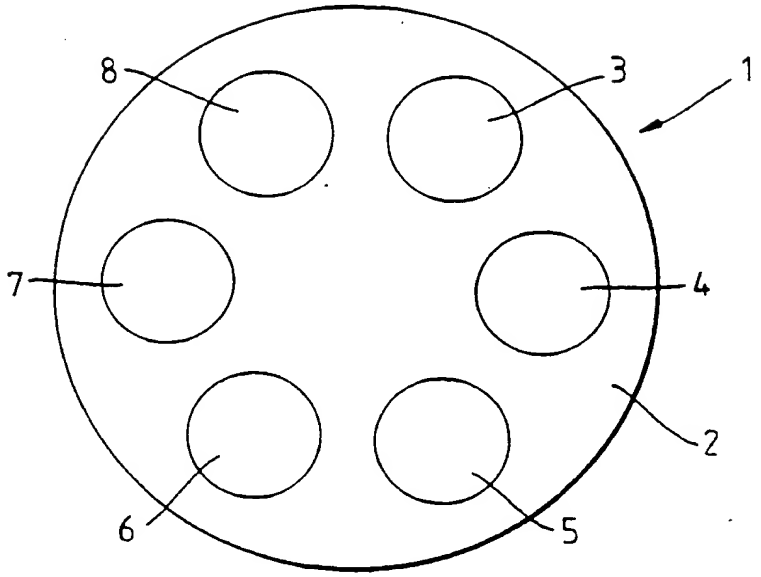


Fig.2.

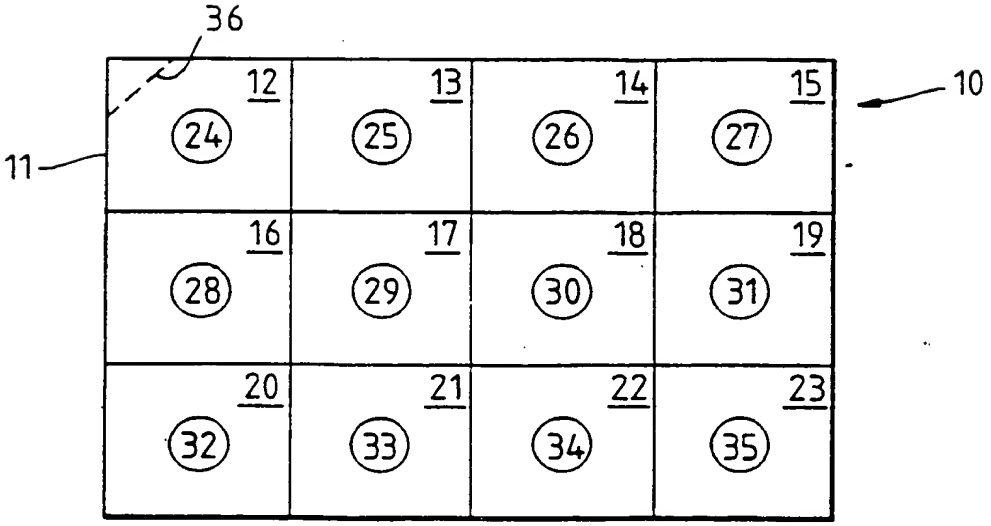


Fig. 3.

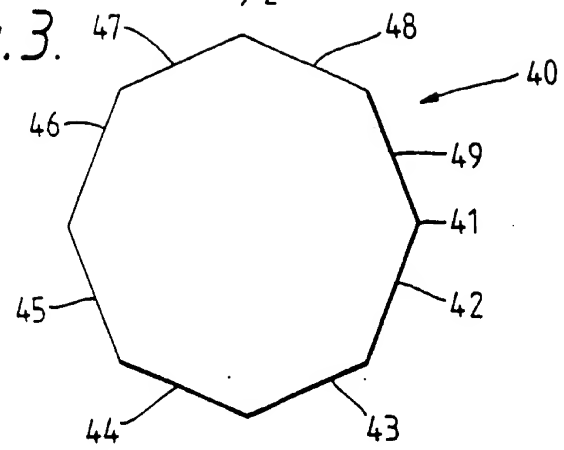


Fig. 4.

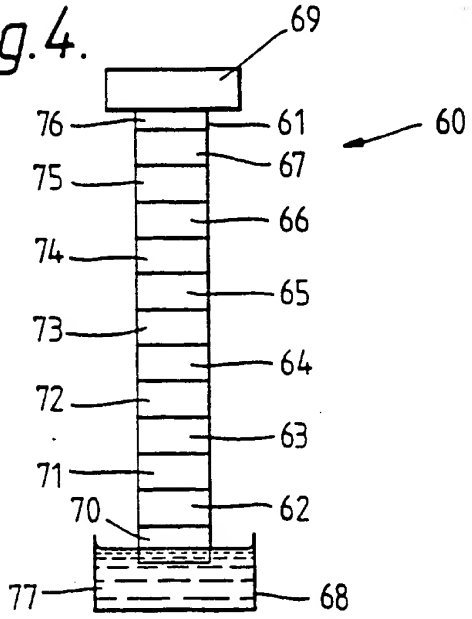
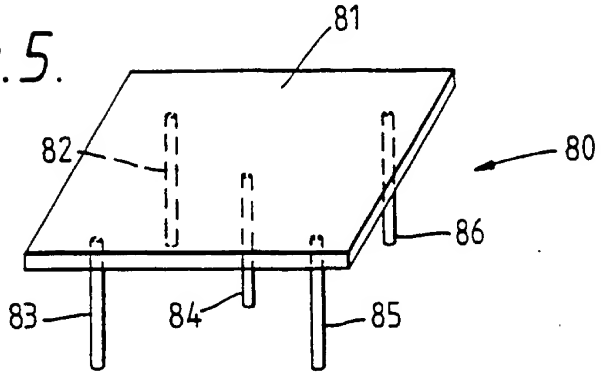


Fig. 5.



INTERNATIONAL SEARCH REPORT

onal Application No
T/GB 93/02454

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 G01N33/537 G01N33/543 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 177 191 (SERONO DIAGNOSTICS PARTNERS) 9 April 1986 ---	
A	EP,A,0 188 093 (IMMUNOMEDICS INC) 23 July 1986 ---	
X	WO,A,92 16838 (GEC-MARCONI LIMITED) 1 October 1992 see the whole document ---	1-22
P,X	EP,A,0 538 053 (GEC-MARCONI LIMITED) 21 April 1993 see the whole document ---	1-22
P,X	GB,A,2 261 949 (GEC-MARCONI LIMITED.) 2 June 1993 see page 15 - page 19 -----	1-22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1994	15. 03. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Cartagena y Abella,P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte[illegible]nal Application No
PCT/GB 93/02454

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0177191	09-04-86	CA-A- 1261745	26-09-89
EP-A-0188093	23-07-86	US-A- 4737453	12-04-88
		AU-A- 5070785	19-06-86
		CA-A- 1254828	30-05-89
		JP-A- 61144573	02-07-86
WO-A-9216838	01-10-92	GB-A- 2255637	11-11-92
EP-A-0538053	21-04-93	GB-A- 2260609	21-04-93
GB-A-2261949	02-06-93	EP-A- 0569574	18-11-93
		WO-A- 9311430	10-06-93

10/27/98

Form PCT/ISA/210 (patent family annex) (July 1992)